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PCR

1. Add the following to each sample:

2.5 μ L 20mM primer 1

2.5 μ L 20mM primer 2

5.0 μ L 10x Buffer (Enzyme-specific)

1.0 μ L pfu enzyme + 5.0 μ L DMSO

OR 0.25 μ L Taq (no DMSO)

1.0 μ L (100ng) cDNA library or genomic DNA

OR 2.0 μ L 2ng/mL plasmids with cDNA inserts

Plus enough 18 M Ω dH₂O to bring each sample up to a final vol. of 50 μ L.

(Must calculate - amount depends on combination of reagents above)

2. Mix a cocktail of the constant reagents – multiply reagent amounts by total num. of reactions +1/2

3. Determine annealing temperature of primers: = (4)(# G-C pairs) + (2)(#A-T pairs) – 5

4. Run PCR at settings:

Step1) 95°C for 3 min

→ Step 2) 95°C for 30 sec

Step 3) X°C for 30 sec X = annealing temp for specific primers

Step 4) 72°C for 1 min/KB **OR** 2 min/KB Taq = 1 min/KB pfu = 2 min/KB

Step 5) 72°C for 5 min

Step 6) 37°C for 30 sec

5. If using plasmids, do 5 cycles of Steps 2-4 w/ Step 3 at (X-5)°C and 15 cycles with Step 3 at X°C

If using genomic DNA, do all 20 cycles of Steps 2-4 w/ Step 3 at X°C

NEXT: RUN PROTEIN GEL & COOMASSIE STAIN OR WESTERN BLOT

NEXT: DNA CLEAN-UP

DNA CLEAN UP

1. Combine all PCR products with same results.
2. Add an equal part phenol-chloroform (stored in 4°C fridge) to the product. Vortex quickly and spin down samples at max speed for 2 min.
3. Pipette off top layer (has the DNA) into a new tube. Discard phenol-chloroform waste in hazardous waste jar.
4. Add 1/10 vol. 3M NaOAc and 3x vol. ethanol.
5. Let samples incubate at room temperature for 5 min.
6. Spin down at max speed for 15 min.
7. Decant all liquid from tube and discard.
8. Add 200µL 70% ethanol to the tube. Invert a few times & spin down at max speed for 3 min.
9. Decant ethanol from tube and let pellet air dry for 10-15 min.
10. Resuspend pellet in 20µL 18 MΩ dH₂O

PREVIOUS : PCR

NEXT : RESTRICTION DIGEST

RESTRICTION DIGEST

Notes: DNA volume should never be more than 25% of total vol. Enzymes volume should never be more than 10% of total vol.

1. Add the following in order:

1.0 μ L DNA

2.0 μ L enzyme-specific buffer

2.0 μ L 10x BSA (if needed for specific buffer)

1.0 μ L Enzyme 1

1.0 μ L Enzyme 2

13.0 μ L 18 M Ω dH₂O

3. Pipette up and down a few times (without introducing air bubbles)

4. Incubate at 37°C for at least 4 hours (Can incubate overnight)

5. Run a 1% agarose gel – See **RUN DNA GEL**

6. Take a picture & cut out desired DNA band using a UV light board and a razor. Store band in a 1.5mL tube at 4°C until ready to use.

PREVIOUS: DNA CLEAN UP

NEXT: GEL PURIFICATION

GEL PURIFICATION

1. Add 3 vol NaI (sodium iodide) to 1 vol of DNA agarose gel of solution and melt at 45-55°C. Invert tubes a few times to speed up melting process. (Ex. Add 300µL for every 0.1g of gel – for 0.2g sample, add 600µL NaI)
2. Take glass milk stock (stored at room temp) and resuspend beads. Takes about 5 min vortexing.
3. Add 5µL glass milk to each sample. Vortex quickly and incubate at room temperature for 5 min on the rotator. (For amounts greater than 5µg DNA, add an additional 1µL glass milk per µg of DNA over 5µg.)
4. Spin down samples at max speed for 5 sec. Decant supernatant and discard.
5. Resuspend pellet in 1mL New Wash. (stored at room temp) (Combine samples now if needed.)
6. Spin down at max speed for 5 sec. Decant supernatant and discard. Make sure to remove all liquid. *Let air dry for 5 min.*
7. Resuspend pellet in 20µL 18 MΩ dH₂O. Incubate at 45-55°C for 5 min. (Removes DNA from beads)
8. Spin down at max speed for 5 sec. Pipette supernatant (has the DNA) into a new tube. Discard remaining pellet. Store at -20°C.

PREVIOUS: RESTRICTION DIGEST

NEXT: DNA LIGATION

OVERNIGHT DNA LIGATION

1. Thaw 10X T4 DNA Ligase Buffer. Set up 1 control (just vector) and x samples (vector + insert).
Want 50-100 ng vector DNA and 250-300 ng insert DNA. Adjust volumes accordingly.

VECTOR + INSERT

3.0µL vector

8.0µL insert

1.5µL 10X T4 DNA Ligase Buffer

1.0µL T4 DNA Ligase

1.5µL 18 MΩ dH2O

VECTOR ONLY

3.0µL vector

1.5µL 10X T4 DNA Ligase Buffer

1.0µL T4 DNA Ligase

9.5µL 18 MΩ dH2O

2. Mix in 1.5mL Eppendorf tube in this order: water, buffer, vector, insert, enzyme. Final Reaction volume should be 15µL.
3. Incubate overnight in a 16°C incubator in cold room.

PREVIOUS: GEL PURIFICATION

NEXT STEP: TRANSFORMATION INTO COMPETENT CELLS

RAPID DNA LIGATION NOTES

Tube #1 – Ligase Buffer

Tube #2 – DNA Dilution Buffer

Tube #3 – T4 DNA Ligase

Incubate ligation on bench for 5 min.

(More elaborate directions are in rapid ligation kit.)

OVERNIGHT (O/N) INOCULATION

Notes: Numbers are for each sample. Multiply by number of samples, make a cocktail & aliquot. Use glass tubes with metal caps for this.

1. Add 2mL LB + 4 μ L specified drugs.

*Want 100 μ g/mL Amp total, so if using 50mg/mL Amp stock, use 2 μ L/mL LB

* Want 34-68 μ g/mL chloramphenical, so if using 34 μ g/mL Chloram stock, use 1-2 μ L/mL LB

Blares, BGLS, & Rosettas– chloramphenical & ampicillin

DH5 α , STBL2 - ampicillin

2. Pick one colony for each sample using a toothpick or 200 μ L pipette tip. Just leave the tip in the LB.
3. Label & grow overnight in 30°C or 37°C shaker depending on cells.
4. Using a wire loop, streak an LB plate w/ appropriate drug, with samples because may need later on. O/N inoculations last less than 24 hours. Grow up O/N in 30° or 37° incubator, depending on plasmid & cells. Store in 4°C.

PREVIOUS: TRANSFORMATION INTO COMPETENT CELLS

NEXT: MINI-PREP

NEXT STEP: MAXI-PREP REST OF O/N INOCULATIONS

MINI-PREP

1. Pour 1.5mL from each 2mL O/N inoculations into eppendorf tubes. Return stock samples to 4°C.
2. Microfuge tubes for 30 sec - 5 min.
3. Decant & discard supernatant using a Pasteur pipette attached to vacuum (if have a lot of samples).
4. Add 100µL Solution I to each sample. Vortex all samples until pellet is resuspended. (Hold 2 together on vortex for more vibration.)
5. Add 200µL Solution II to each sample. Mix gently by inverting a few times.
(For 5mL Sol. II: 100µL 10N NaOH, 4.4mL 18 MΩ dH₂O, 500µL 10% SDS)
6. Add 150µL Solution III to each sample. Vortex inverted briefly. Incubate on ice for 5 min.
7. Spin down samples for 10 min. Decant supernatant into a new tube & discard pellet.
8. Add 1mL ethanol to each sample. Invert a few times to mix.
9. Spin down at max speed for 10 min. Discard supernatant.
10. Add 200µL 70% ethanol. Invert gently a few times.
11. Spin down for 2 min. Discard supernatant.
12. Keep tubes turned upside down & let pellets air dry for 10-15 min. (Pellets turn clear when dry)
13. Resuspend pellets in 38µL 18 MΩ dH₂O + 2µL 10mg/mL DNase-free RNaseA

PREVIOUS: O/N INOCULATION

NEXT : RESTRICTION DIGEST

NEXT STEP: MAXI-PREP REST OF O/N INOCULATIONS

RUNNING A DNA GEL

1. Set up gel container & place in 1 or 2 combs.
2. Pour a 1% agarose gel. If mixture is already made, microwave for about 45 sec. (or until melted), then let cool until can place hand comfortably on glass. If mixture is not made, mix 0.4g agarose with 40mL TAE buffer. Microwave to dissolve & let cool until can touch container with bare hand. **Swirl in 2-3 μ L EtBR** and then pour gel. Let gel cool at room temperature for 15-20 min. (Pour gel while caster is in 4°C & let set there if want it to set faster.)
3. Set up gel and make sure that there is enough TAE buffer to cover the top of the gel. Place as many dots of gel loading dye as you have samples on a small piece of Para film. Pipette samples up and down to mix in the dye and then load the appropriate lane. Fill lanes – add 2 μ L 1 KB DNA Ladder to first lane.
4. For PCR products, add 6 μ L sample. For Restriction Digests, add 20 μ L sample.
5. If running half of a gel, run the bottom part 1st. Only run for 15 min at 100V. Run the top 1/2 for 20 min. at 100V for best results.
6. Run a full-length gel if really need to spread out the bands. Can run full length for 40 min at 80V.
7. Take a picture of gel using UV light. Cut out desired DNA band if needed.

PREVIOUS: RESTRICTION DIGEST

NEXT: GEL PURIFICATION

PREVIOUS: MINI-PREP

NEXT: MAXI-PREP

*Usually add 5 μ L EtBr to 100mL gel – if making less gel, cut back the EtBr proportionally.

ISOPURE MAXI-PREP

1. Make up LB stock in 1L flasks. For High Copy Plasmid (ex. pBS, pLD1, p90), **use 100mL LB**. For Low Copy Plasmid (ex. pET14B), use 200mL LB. Autoclave & let cool.
2. Add drug as needed for specific plasmid. Add **2 μ L 50mg/mL stock Ampicillin per mL LB**, so have final conc. of 100 μ g/mL amp. Add **1 μ L 34mg/mL stock Choramphenical per mL of LB**, so have final conc. of 34 μ g/mL. (Ex. For 100mL LB – add 200 μ L amp and/or 100 μ L chloram)
3. Add rest of mini-prep O/N Culture (~0.5mL) directly to LB culture. Grow up O/N in 30° or 37° C shaker depending on cells.

Blares: 37°C	Rosettas: 37°C	STBL2: 30°C
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4. Make glycerol stock of O/N culture. Add 850 μ L culture & 150 μ L glycerol to a 1.5mL screw top tube. Mix. Label well & wrap clear tape over label to ensure that it doesn't fade. Store at -80°C.
5. Pour culture into 250mL white centrifuge tubes and centrifuge at ~10,000 rpm for 10 min with the JA-14 rotor. Decant supernatant & discard.
6. Resuspend pellet in 10mL buffer A by pipetting. (Make sure RNase was added to buffer A.)
7. Transfer to a 50mL centrifuge tube. Add 10mL buffer B. Mix gently by inverting tube 10 times. (Do not vortex!) Let mixture stand at room temp for 5 min. (Should become clear & viscous)
8. Add 13mL buffer C1 to tube. Invert tube 10 times to gently mix. (White precipitation should appear) (Caution: buffer C1 contains chaotropic agent –handle with care.)
9. Centrifuge for 10 min at 13,000 rpm at 4°C.
10. Transfer supernatant to another 50mL centrifuge tube. Keep tube on ice.
11. Transfer ½ of supernatant to the DNA binding column unit. Centrifuge the column at 4,000 rpm for 5 min in clinical centrifuge.

12. Carefully remove DNA binding column from the unit and discard the pass-thru from the collection tube. Reassemble the DNA binding column unit.
13. Repeat steps 11-12 for the remaining supernatant using the same DNA binding column.
14. Add 20mL 70% ethanol to the DNA binding column unit. Centrifuge for 5 min. at 4,000rpm in the clinical centrifuge. Remove the DNA binding column unit, discard the pass-thru, and reassemble.
15. Repeat step 14.
16. Centrifuge the unit (dry spin) for 10 min at 4,000rpm in the clinical centrifuge.
17. Open the cap and let the unit stand at room temp. for 10 min. to dry leftover ethanol.
18. Transfer the DNA binding column to a new 50mL centrifuge tube. Add 1 ml preheated (65-70°C) 18 MΩ dH2O to the center of the DNA binding column and let stand at room temp for 1 min.
19. Elute DNA by centrifuging the unit for 5 min. at 4,000 rpm in the clinical centrifuge.
20. Repeat steps 18-19 for maximum efficiency. (get ~50% more DNA if repeat) Combine eluted DNA in 2mL tube.
21. Dilute 3μL DNA into 597μL 18 MΩ dH2O (= 200 fold dilution) Determine OD₂₆₀ using RNA/DNA program on Spectrometer. Calculate concentration using Beer's Law, as follows:

$$C = [A * df * E] / \lambda \qquad C = (A * 200 * 50) / 1 \qquad C = (10,000 * A) \quad \text{dil. factor}=200$$

$$A = \text{absorption} = \text{OD}_{260} \qquad C = (A * 100 * 50) / 1 \qquad C = (5,000 * A) \quad \text{dil. factor}=100$$

df = dilution factor = 200 or 100 or whatever you dilute by

E = epsilon = 50μg / mL*cm λ = path length = 1 cm C = concentration =ng/μL

PREVIOUS: O/N INOCULATION, MINI-PREP, DNA GEL

NEXT: TRANSFORMATION INTO COMPETENT CELLS

QIAGEN MAXI-PREP

1. Make up LB stock in 1L flasks. For High Copy Plasmid (ex. pBS, pLD1, p90), **use 100mL LB**. For Low Copy Plasmid (ex. pET14B), use 200mL LB. Autoclave & let cool.
2. Add drug as needed for specific plasmid. Add **2 μ L 50mg/mL stock Ampicillin per mL LB**, so have final conc. of 100 μ g/mL amp. Add **1 μ L 34mg/mL stock Choramphenical per mL of LB**, so have final conc. of 34 μ g/mL. (Ex. For 100mL LB – add 200 μ L amp and/or 100 μ L chloram)
3. Add rest of mini-prep O/N Culture (~0.5mL) directly to LB culture. Grow up O/N in 30° or 37° C shaker depending on cells.

Blares: 37°C	Rosettas: 37°C	STBL2: 30°C
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4. Make glycerol stock of O/N culture. Add 850 μ L culture & 150 μ L glycerol to a 1.5mL screw top tube. Mix. Label well & wrap clear tape over label to ensure that it doesn't fade. Store at -80°C.
5. Pour culture into 250mL white centrifuge tubes and centrifuge at ~10,000 rpm for 10 min with the JA-14 rotor. Decant supernatant & discard.
6. Resuspend pellet in 10mL Buffer P1 (Stored at 4°C). Transfer into a 50mL centrifuge tube.
7. Add 10mL Buffer P2 (Stored at room temp.). Invert a few times to mix.
8. Add 10mL Buffer P3 (Stored at room temp.). Invert until turns a cloudy white and “mucus like”. (Takes about 15-30 sec.)
9. Incubate on ice for 10 min. Centrifuge at ~15500 rpm (34000G) for 20 min with the JA-17 rotor.
10. Set-up 30mL filter tip on top of 500mL flask. Pour 10mL QBT Equilibrium Buffer into the top & let filter through. (Takes about 10 min.)
11. Pour supernatant through 2 layers of cheesecloth into tip & let filter through. (5-20 min.)
12. Fill tip to top (30mL) QC Wash buffer & let filter through. (Takes about 20 min.)

13. Get new centrifuge tube & add 10 1/2 mL isopropanol (labeled 2-propanol) to it. Once tip is done filtering, place on top of new tube and pour in 15mL QF Elution buffer. Let filter through. (Takes about 10 min.)
14. Incubate in -20°C freezer for a minimum of 2 hours, but can leave overnight.
15. Centrifuge for 30min. at ~17,000 rpm using the JA-17 rotor.
16. Pour off supernatant & add 5mL 70% ethanol. Invert a few times.
17. Centrifuge at ~17,000 rpm for 30 min with the JA-17 rotor.
18. Decant supernatant and let pellet air-dry. (Takes 10 –15 min.)
19. Resuspend pellet in 100-200µL 18 MΩ dH2O. Put into a 1.5mL tube.
20. Dilute 3µL DNA into 597µL 18 MΩ dH2O (= 200 fold dilution)
21. Determine OD₂₆₀ using RNA/DNA program on Spectrometer. Calculate concentration using Beer's Law, as follows:

$$C = [A * df * E] / \lambda \qquad C = (A * 200 * 50) / 1 \qquad C = (10,000 * A) \quad \text{dil. factor}=200$$

$$A = \text{absorption} = \text{OD}_{260} \qquad C = (A * 100 * 50) / 1 \qquad C = (5,000 * A) \quad \text{dil. factor}=100$$

df = dilution factor = 200 or 100 or whatever you dilute by

E = epsilon = 50µg / mL*cm λ = path length = 1 cm C = concentration =ng/µL

PREVIOUS: O/N INOCULATION, MINI-PREP, DNA GEL

NEXT: TRANSFORMATION INTO COMPETENT CELLS

RUNNING A PROTEIN GEL

1. Add 2X sample buffer if not already added. Heat shock samples for 30 sec in 100°C heat block. Load appropriate gel (or gels) into container. Use gel dam if only running 1 gel.
2. Fill center area to top with buffer. Fill container w/ ~ 1 inch buffer. Remove combs.
3. Load gels with 5-6µL sample for each column. Load 3µL mwm (molecular weight marker) in 1st column.
4. Double check that buffer covers top of gels.
5. Attach electrodes and run at constant A = 70mA until through stacking gel, then can speed up.
If running 2 gels – start at 100mA.
If running 4 gels – start at 140mA

NEXT STEP: COOMASSIE STAIN OR WESTERN BLOT

COOMASSIE STAIN

1. Remove gel using razor blade and place in tip cover with about 1/4” coomassie stain.
2. Cover with saran wrap and place on rotator for 1-2 hours.
3. Pour off stain into “Used Coomassie” jar. Rinse gel 1-2x with water.
4. Pour in a little 10% acetic acid, swish around, and pour off.
5. Refill with about 3/4” 10% acetic acid. Wad up a few kimwipes and place in corner to speed up de-staining time. Occasionally refresh destaining solution.
6. Place on rotator until enough stain has seeped out of the gel. (Takes 5-10 hours) Take picture & label. Make sure the gel is facing the right way!

WESTERN BLOT PREP FOR *DICTYOSTELIUM*

1. Resuspend cells in media if plated. Take 6-8mL aliquots depending on density of cells and pipet into 15mL tube. (Scrap ~ 1 1/2 inch colonies from an SM-5 plate and skip to 4.)
2. Take 9.5μL sample from tube and count cells using a hemocytometer. Multiple cell number by volume taken to obtain total cells in the sample. (This final number = C, see below)
3. Spin down samples 4,000 rpm for 5 min in microcentrifuge. Remove supernatant.
4. Resuspend cells to appropriate density with 10mM Tris (pH 7.5). You want the final concentration of cells/mL to be around 10^8 . Use the equations below to calculate the amount needed.
→Multiply cell count by total mL you are spinning down. This equals the total amount of cells.
$$(\text{Cell Count}) \times (\text{mLs}) = \text{Total Cells}$$

→Divide this total cells number by 10^8 to determine how many mLs 10mM Tris to resuspend in.
$$(\text{Total Cells}) / 10^8 = \text{mL 10mM Tris to use so that your final conc. will be } 10^8 \text{ cells/mL.}$$
5. Transfer 10 μL from sample tubes into new tubes. These new tubes are now the Cell Lysate Tubes. Freeze in liquid N₂, thaw, freeze again, and thaw again to lyse the cells. Mix well and use 1-3μL in Bradford Assay
6. To the original sample tube – add an equal volume (remembering to subtract the 10μL you just took out) of 2X Sample Buffer and immediately boil for 10min.
7. Do a **Bradford Assay** with your Bradford Assay Sample Tubes to get the most accurate protein concentration.
8. Set up a protein gel. Add 3μL mwm to first lane. (See Running a Protein Gel for more details)

PREVIOUS: CELL CULTURE

**NEXT: WESTERN BLOT OR
BRADFORD ASSAY**

BRADFORD ASSAY

1. Thaw tube of 10mg/mL (100x) BSA (stored in -30°C). **First set up your standards.** Take out 7 tubes and label 0, 1.9, 3.75, 7.5, 15, 30, 60. Add 800µL 18 MΩ dH₂O to Tube 0. Add 788µL 18 MΩ dH₂O to Tube 60. Add 400µL to the other 5 tubes.
2. Add 12µL 10mg/mL BSA to Tube 60 (for a total of 120µg). Vortex quickly. Take 400µL out of tube 60 and add to tube 30. Vortex tube 30 quickly. Now have 60µg total in tube 60. Take 400µL out of tube 30 and add to tube 15. Vortex quickly. Take 400µL out of tube 15 and add to tube 7.5. Vortex quickly. Take 400µL out of tube 7.5 and add to tube 3.75. Vortex quickly. Take 400µL out of tube 3.75 and add to tube 1.9. Vortex quickly. Take 400µL out of tube 1.9 and discard. Now add 400µL dH₂O back to all standards tubes **except 0** to raise volume to 800µL.
3. Add 200µL Biorad Assay (Red Dye) (stored in 4°C) to each standard. Invert each one a few times. You should see a colorimetric range now.
4. **Now set up your Bradford samples.** Take out a new tube for each sample. Label these tubes and add 800µL dH₂O and 200µL Biorad Assay (Red Dye) to each. Add 1-3µL of Cell Lysate. Add the same amount of 10mM Tris to your standard tubes to match. **Record amount added. This amount is now Q in equation below.**
5. Perform spectrophotometer analysis on all tubes. Use λ program with wavelength 595nm. Use Tube 0 to blank spec. Then read Tube 0 as sample. Read rest of standards in order. Then read samples.
6. Print out summary and graph standards using Kaledagraph. Last two points (30&60) may start saturating and may throw off the line. Discard these points if that is the case. Fit a line to the points and use the equation to solve for concentration of samples. $Y = mx + b \rightarrow X = (Y - b) / m$
Solve for X. This answer is the µg in **Q** µL. Divide by Q to get **M** µg/µL. However, now remember that you diluted the sample by 2 when you added sample buffer. So now divide **M** by 2 to get the concentrations (µg/µL) of your samples that are mixed with sample buffer.

PREVIOUS: WESTERN BLOT PREP

NEXT: RUN A PROTEIN GEL

WESTERN BLOT

1. Remove protein gel from plate and place in container with ~ 1/2" 1X SD Transfer Buffer. Place two absorption pads in a different container with same buffer. Soak 10-30 min.
2. Set up Trans-Blot SD. Cut appropriate sized membrane and label front right upper corner with a pencil. Wet in 18 MΩ dH2O water. Place on absorption pad on Trans-Blot. Place membrane labeled side up next. Then place gel on top of membrane and add last absorption pad on top. Follow directions to run the Trans-Blot for designated time. (15V for ~20 min.)
3. Remove membrane, mark mwm bands with a pencil. Throw away gel and blot pads. Place membrane in a container with 50mL 5% milk/PBT. Place on rotator for 2 + hours. (Can leave overnight here or at step 4, if add 30μL 10% 1.54M sodium azide or seal in bag)
(PBT = PBS with 0.2% Tween. 1L PBT = 10mL 20% Tween, 100mL 10X PBS, 890mL dH2O)
(Add 2.5g dry milk to 50mL PBT – mix well. Add 1g dry milk to 20mL PBT)
4. Add determined dilution of primary antibody to fresh 20-50mL milk/PBT solution. Let rotate 1+ hours. If leave O/N, seal in bag.
For dynacortin: 1:50,000 **suc pAb**- If have 50mL milk/PBT, add 1μL **suc pAb**.
For myosinII: Add 5μL **myo6Ig** to 20mL milk/PBT.
5. Rinse membrane 3x with PBT. Do 4 - 5min washes with PBT on rotator.
6. Pour off PBT and refill with secondary antibody in 20mL milk/PBT. Let rotate 1+ hours.
For dynacortin: 1:5,000 dilution of **gar HRP**. If 20mL milk/PBT, add 4μL.
For myosinII: Add 5μL **gam** (goat anti –mouse) to 20mL milk/PBT.
7. Rinse membrane 3x with PBT. Do 4 - 5min washes with PBT on rotator.
8. Shake off excess PBT and wrap membrane in sheet protector. Mix 500μL ECL Reagent 1 with 500μL ECL Reagent 2. Pour mixture onto membrane and wait 1 min. Tilt membrane and pour off excess liquid. Expose using Chemiluminescence setting on VersaDoc.

TRANSFORMATION INTO *DICTYOSTELIUM*

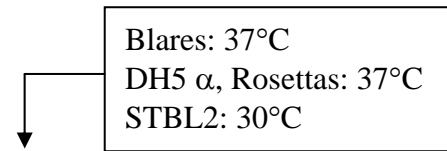
(Use Transformation into Dictyostelium Worksheet)

1. Resuspend enough parent strain cells (combine in 50mL tube if needed). Take cell count for each.
(Need 1×10^7 cells per 350 μ L for each transformation)
2. Multiply cell count by total mL to get total cell count. Perform calculations below:
Total Cells / $1 \times 10^7 = X$ $X * 0.35\text{mL} = Y = \text{amt E-Pore buffer needed in Step 6.}$
3. Spin down cells at 1,500rpm for 5 min. Get a bucket of ice now.
4. Decant supernatant and resuspend pellet in 10mL ice-cold E-Pore Buffer. Keep on ice.
5. Spin down cells at 2,000rpm for 5 min. Label desired number of cuvettes and keep on ice until ready to use. Label 1 petri dish for each cuvette. Chill Enriched + PennStrep Only 1.5X HL-5 media in 4°C.
6. Resuspend in calculated amount of ice-cold E-Pore Buffer (Must have 1×10^7 cells per 350 μ L E-Pore Buffer) Aliquot 350 μ L of cells in E-pore buffer into each cuvette. Keep on ice.
7. Add appropriate amount of DNA or plasmid to each cuvette. Keep on ice.
8. Gather cuvettes, petri dishes, chilled media, & pipets. Go to cell culture room. Add 9mL media to each petri dish. Make sure to use sterile conditions.
9. To electroporate cells: Set cap knob to 3.0. Set voltage @ 1.3kV for orf+ cells, myoII, 1.1kV for 11-5.1 cells . Hold down both red buttons until hear a beep, release buttons. τ should be between 1.5 – 2.0. Pour contents of cuvette into matching petri dish. Shake back and forth and then side to side gently to spread cells out. Let grow overnight in no-drug media.
10. Pour off media and carefully drip in 9-10mL drug selection media. **Do not resuspend cells.**
11. Change media or split every 2-3 days. Do not shake plate for 1st change, then shake front→back and side→ side a few times for the rest of the changes. (Do not swirl in a circle.)

TRANSFORMATION INTO COMPETENT CELLS

1. Take out an aliquot of competent cells from -80°C and immediately put on ice.
2. Thaw DNA. Use 1 – 3ng DNA per sample depending on design of experiment. Set up enough tubes for total samples plus 1 No DNA control. (*Use $2\mu\text{L}$ ligation rxn, $1\mu\text{L}$ pUC*)
3. Add 50-100 μL competent cells to each sample and the control.
4. Add specified amount of DNA to each of the sample tubes. Mix by pipetting up and down a few times.
5. Leave tubes on ice for 30 min. Prepare 42°C heat block by adding a little 18 M Ω dH₂O into wells that will be used. Let temp of the water get to 42°C
6. Heat shock tubes at 42°C for 40 sec. (Exceptions: STBL2 for 28 sec, DH5 α for 45 sec)

7. Put back on ice for 5 min.



8. Add 1mL LB to each tube. Put all tubes together and, depending on strain, place in 37°C shaker for 45 min or 30°C shaker for 90min.
9. Spin down cells for 30 sec - 5 min at max speed. Remove enough LB so there is 100-200 μL left.
10. Resuspend cells in remaining 250 μL LB & transfer to plates with appropriate drugs.

pRARE – chloram resistant	Rosetta cells – no drug resistance
pakA – amp resistant	STBL2 cells – no drug resistance
dynaFL, C181, N173, ΔCHD , & C181 ΔCHD – amp resistant	BLR(DE3) cells – tet ^r resistant
11. Grow up overnight in 30°C or 37°C incubator depending on cells.

PREVIOUS: MAXI-PREP

NEXT: TESTING EXPRESSION

TESTING EXPRESSION

Notes: Numbers are for each sample. Multiply by number of samples, make a cocktail & aliquot. Use glass tubes with metal caps for this.

1. Add 2mL LB + 4 μ L amp + 4 μ L chloramphenical to each tube. (Make sure correct drugs for plasmid – may only need chloram)
2. Pick one colony for each sample using a toothpick or 200 μ L pipette tip. Just leave the tip in the LB.
3. Label & grow overnight in 37°C shaker.
4. Add 3mL + 6 μ L amp + 6 μ L chloramphenical to all new tubes. Make glycerol stocks from overnight cultures by adding 850 μ L culture + 150 μ L glycerol. Label & store in -80°C.
5. Inoculate with 100-500 μ L of the overnight-saturated samples (depending on density).
6. Incubate new samples in 37°C shaker for 1-3 hours. Remove when OD₆₀₀ is between 0.6-1.0. Test this in the Spectrophotometer using a blank of 400 μ L LB and 400 μ L of each sample.
7. Add 12 μ L 100mM IPTG to each 3mL sample. (Want 0.4mM IPTG in sample.)

PREVIOUS: TRANSFORMATION

NEXT: COLLECT TIME POINTS

COLLECT TIME POINTS

1. Collect 200 μ L of each sample & spin down at max speed for 3 min.
2. Remove LB and resuspend in 40 μ L 2X Sample Buffer.
3. Boil for 5 min. in 100°C heat block.
4. Freeze samples in -20°C until ready to run a protein gel.

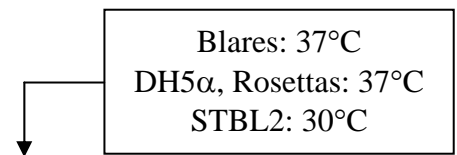
MAKE COMPETENT E. COLI CELLS

1. Make SOB & autoclave. Make TB & store at 4°C. Autoclave ~4-6 bottles. Autoclave tubes, & microfuge tubes as needed.
2. Add 2.5mL 1M MgCl₂ to 250mL SOB right before inoculation.
3. For STBL2 – do not add any drug. For Rosetta – add 500µL 34µg/mL chloramphenical For Blares & BGLS – add 500µL 34µg/mL chloramphenical. DH5α – do not add any drug
4. NEED VERY STERILE CONDITIONS: Using a wire loop, transfer 10-12 colonies to a 1mL tube of SOB & pour into beaker with 250mL SOB culture.
5. Grow at 22°C (room temp.) in shaker until A₆₀₀ is between 0.6-0.8. Test this using a blank of 400µL SOB and samples of 400µL in the Spectrometer.
6. When samples are ready, pour broth into sterile centrifuge bottles and put on ice for 10 min. Spin at 2,500g (6,000 rpm) for 10 min. Remove supernatant.
7. Using a 25mL pipette, resuspend pellet in 20mL ice-cold TB. Add 60mL TB to make final vol 80mL
8. Put samples on ice for 10 min. Spin at 2,500g for 10 min. (Get liquid nitrogen now.)
9. Pour off supernatant & resuspend in 20mL TB.
10. Swirl in 1.4mL DMSO to make final [DMSO] = 7% & incubate on ice for 10 min.
11. Aliquot 500µL into 1.5mL tubes & quickly freeze in nitrogen. Store at –80°C. Should last 40 days.

NEXT: TEST COMPETENCY OF E. COLI CELLS

TEST COMPETENCY OF E. COLI CELLS

1. Take out DNA and 1-500 μ L aliquot of each line of cells testing, thaw and place in ice.
2. Label 1mL tubes for 1 control & 2 tests for each line of cells. Add 1 μ L DNA to each sample tube.
(Not the control tube.)
3. Add 100 μ L cells to each tube. Mix by pipetting up and down gently.
4. Leave tubes on ice for 30 min. Prepare 42 $^{\circ}$ C heat block by adding a little 18 M Ω dH₂O into wells that will be used. Let temp of the water get to 42 $^{\circ}$ C
5. Heat shock tubes for 40 sec. (Exceptions: STBL2 – 28sec, DH5 α – 45 sec)
6. Put back on ice for 5 min.
7. Add 1mL LB to each tube. Put all tubes together and, depending on strain, place in 30 $^{\circ}$ C for 90 min. or 37 $^{\circ}$ C shaker for 45 min.
8. Take out samples & dilute by taking 110 μ L from 1st tube and adding to 1mL LB in a new tube. The first tube becomes [0.9] and the second becomes [0.09]. Mix the second well and pipette 110 μ L into a new tube with 1mL LB. This third tube is now [0.009]. Mix well.
9. Spin down cells for 5 min at max speed. Remove 850 μ L LB and discard.
10. Resuspend cells in remaining 250 μ L LB & transfer to plates with appropriate drugs.
11. Grow up overnight in 30 $^{\circ}$ C or 37 $^{\circ}$ C incubator.



PREVIOUS: MAKE COMPETENT CELLS

PROTEIN PURIFICATION

Notes: Day 1 – Step 1 Day 2 step 2-12 Day 3 – Steps 12 - 21 or 22

1. Inoculate 250mL LB + 500 μ L amp + 500 μ L chloram with 10-12 colonies of desired protein. Let grow up to saturation overnight in 30° or 37°C shaker.
2. Make glycerol stock of the O/N cultures by adding 850 μ L culture to 150 μ L glycerol in a screw-top tube. Label well and cover label with clear tape to avoid fading. Store in -80°C freezer.
3. Inoculate as many 2L aliquots of LB + 4mL amp + 4mL chloram as need. Aliquots should be in 6L flasks. Let incubate in 30° or 37°C shaker until in log phase.
4. Test absorbance of cultures until A600 is between 0.6-1.0. When cultures are in log phase, add 0.19g IPTG resuspended in 2mL 18 M Ω dH2O to each 2L culture.
5. Take 200 μ L samples at T0, & return cultures to incubator.
6. Spin down To samples for 3 min., remove sup, resuspend in 40 μ L 2X Sample Buffer, boil for 5 min. & store in -30°C until ready to run a protein gel.
7. Remove cultures at designated time (usually T3-T4 is best). (Take time point sample and repeat step 6.) Spin down cultures at 4,000rpm for 10min. in 1L centrifuge bottles using the JLA-8.1 rotor. If have too many samples, spin 6, pour off the supernatant, add more culture, and spin again.
8. Pour off the supernatant, wash pellet quickly in a little 10mM Tris (pH 7.5). Resuspend pellet in 25mL 10mM Tris per Liter of original amount of culture. (Ie. If spun down 2 L of cells in one bottle, add 50mL 10mM Tris.)
9. Switch cells to the 250mL centrifuge bottles if possible & spin down at 12,000 rpm for 10-15min using the JA-14 rotor. (Get liquid nitrogen now. Make lysis buffer now.)

10. Pour off supernatant & resuspend pellets in 30mL Lysis Buffer per Liter of original amount of culture. (See Recipes to make Lysis Buffer.)
11. Freeze 30mL aliquots in small centrifuge tubes in liquid nitrogen. Store in -80°C overnight.
12. Thaw tubes of protein in tub of luke warm water – important that protein does not get above 4°. Keep turning tubes to ensure even thawing. Add fresh protease inhibitors if needed.
13. Pour cells into 1-2 glass beakers and sonicate. Do 2-3 pulses for 30 sec each. Do not touch the sides or bottom of the beaker with the sonicator. Use earphones to protect ears.
14. Pour cells evenly into centrifuge tubes. (Solution should be mucus-like.) Centrifuge for 25 min at 13,000 rpm (~20,000 G).
15. Pour supernatant into a graduated beaker & note amount. Pour into glass beaker with stir bar.
16. Slowly add polyethylenimine (PEI), so that have a 0.2% concentration in supernatant. Must drip in so no local high concentrations. Calculate using:

$$(5\%)(X \text{ mL}) = (2\%)(Y \text{ mL}) \quad X = (2*Y) / 5 \quad Y = \text{amt supernatant} \quad X = \text{amt to add}$$
17. Let stir on med-high speed for 5 minutes. (Should see thick white wisps forming.)
18. Centrifuge for ~5-15 min at 13,000 rpm (~20,000 G). Want 100,000g·min. So if spinning at 20,000g, spin for 5 min. Do not want to spin much more – not good for sup. **Supernatant should come out very clear and almost colorless.** If not, spin a little longer.
19. Pour supernatant into a graduated beaker & note amount. Pour into glass beaker with stir bar.
20. Add enough ammonium sulfate to raise concentration to 45%. Must add SLOWLY so that no local high conc. Add while stirring. Let stir for 5 min after having added the whole amount.

$$[45\%] = 277\text{g/L ammonium sulfate} \therefore \text{Amt to add (g)} = (0.277 \text{ g/mL} * V \text{ mL}) \quad V = \text{amt supernatant}$$

21. Centrifuge for 10 min at 13,000 rpm (~20,000 G). Keep the pellet, but pour off the supernatant into a clean beaker in case the pellet slips.
22. Resuspend pellet in specified amt of No Salt A & run Protein Specific Sizing Column (See Sizing Column Protocol)

dynFL	Resuspend pellet in 8mL No Salt A Run on S300 Column (can only load up to 12mL)
N173	Resuspend pellet in 10mL No Salt A / 1 L of original culture Run on a SP sepharose column
C181	Resuspend pellet in 10mL No Salt A / 1 L of original culture Run on a Nickel Column
23. Run protein gel to identify fractions with the protein if needed.
24. Pool fractions that have the protein & dilute 3 fold. Run Mono S column. (See Mono S Protocol).

NEXT: RUN PROTEIN SPECIFIC COLUMN & PROTEIN DIALYSIS

PROTEIN DIALYSIS

1. Pool desired fractions (run gel to determine fractions if necessary)
2. Cut a piece of dialysis tubing. Clip one end and fill with 18 MΩ dH₂O to check for leaks.
3. Carefully pour protein into a dialysis bag. Clip the other end closed.
4. Make up dialysis buffer in a 2L graduated cylinder with a large stir bar at the bottom.
5. Float bag in dialysis buffer for at least 5 hours – better to leave overnight – in the cold room on a stir plate. Check on it a few times before leaving for night because stir bar sometimes gets off track.
6. When protein reaches equilibrium with buffer, collect protein & fill a 50mL conical with the used dialysis buffer. Label & store buffer in 4°C for use in sedimentation experiments.
7. Determine concentration using λ function on the spectrometer. Dilute 4 μ L protein in 396 μ L 18 MΩ dH₂O for a 100-fold dilution. Dilute 4 μ L dialysis buffer in 396 μ L 18 MΩ dH₂O for a 100-fold dilution. Use 290 as the wavelength and blank against the diluted dialysis buffer. Aliquot protein depending on concentration.

LOW SPEED SEDIMENTATION EXPERIMENT

1. Take out protein samples and spin at 100,000g for 15 min. Make F-actin now.
2. Dilute F-actin to desired concentration with 1X F Buffer. Dilute protein as needed with Dialysis Buffer. Final volume = 50 or 100 μ L for each reaction.
3. Set up reactions. Want final volume of each reaction = 50 μ L. (The setup below is for 10 μ M F-actin and 34 μ M protein. If molarities are different, must recalculate new amounts using concentrations below. Dialysis amount = 40-(Protein μ L). Tube 1 = actin background control. Tube 2 = protein background control.

Reaction Tube	F-actin Conc.	F-actin μ L	Protein Conc.	Protein μ L	Dialysis Buffer μ L	1X F Buffer μ L
1	2	10	0	0	40	
2	0	0	8.0	11.76	28.24	10
3	2	10	0.5	0.74	39.26	
4	2	10	1.0	1.47	38.53	
5	2	10	1.5	2.2	37.80	
6	2	10	2.0	2.94	37.60	

4. Mix the protein with the dialysis buffer first. Then add the F-actin or 1X F Buffer. Pipette gently to mix. Avoid bubbles.
5. Incubate at room temp for 30 min. Then spin down for 25 min at 48,000rpm in the TLA-100 for 100,000 x g or spin at 12.7rpm in the microcentrifuge for 10,000 x g.
6. Decant sup into new 1.5mL tubes. Add 50 μ L 2X Sample Buffer to each tube and boil for 10 min. Each sup sample has a final vol. of 100 μ L & each pellet sample has a final vol. of 50 μ L.
7. Run a protein gel using 5 μ L mwm, 10 μ L sup samples, & 5 μ L pellet samples. Gels can saturate with protein – may have to load less into gel or dilute samples 5 to 10 fold. Run gels at 0.7A for 50 min or more if running more than 1 gel at a time.
8. Dye gels with Coomassie stain. Let stain for at least 5 hours (o/n is better), destain thoroughly by washing gels 3-4 times with 10% Acetic Acid. Each wash should last about 30-60min. Want very clean staining and destaining, so do not microwave – let stain slowly.
9. Take a picture of each gel with the F-Stop at 8, and the focus as close as possible. (~40 for 15 well protein gels.) Set the gamma at 1.0, make the white as high as possible, and make the black = 0. Save the pictures on zip disk & print out a copy.
10. Pick Analysis Tools to do densitometry on the bands. Check the invert box, and set the number of lanes to 2 and the width as large as possible. Once the box is around the first two lanes, click on Auto Grid. Set horizontal baseline and move to the base of the peaks. Fix the lane widths and record the area for each peak.

FALLING BALL VISCOMETRY EXPERIMENT

1. Determine Final Volume, Final [Salt], and Final [F-Actin] needed.

Controls:

1. Actin + 1X F-buffer	2. Protein + 1X F-Buffer
3. 1X F-Buffer	4. Water alone
5. Glycerol alone	

2. Glycerol & water controls are standards. Determine single best angle, size ball, and size tube to use with both of these. Start with 50° or 80°. Must be able to time ball going through water and must be able to get the ball to go through the glycerol.
3. Determine what concentrations and amounts are needed for all reactions and controls. For example, have Final Vol = 50μL, Final [Salt] = 1X, Final [F-actin] = 24μM. Protein = dynacortin. (Since dynacortin acts as a dimer in order to have 1 actin : 1 dimer, must have 2 times amount of dynacortin monomer concentration as actin.) Run a dilutions series for the varied protein to get the widest range of information.

Rxn	Actin : Protein Ratio	[Actin]	[Protein]	[Salt]	μL 10X F-Buffer	μL 106.7 μM Protein	μL 53.3 μM Actin	μL 1.8X F-Buffer
C1	1 : 0	24	0	1X	5	0	22.5	27.5
C2	0 : 1	0	48	1X	5	22.5	0	27.5
C3	0 : 0	0	0	1X	50	0	0	0
1	1 : 1	24	48	1X	5		22.5	0
2	1 : 0.1	24	4.8	1X	5		22.5	0
3	1 : 0.01	24	0.48	1X	5		22.5	0
4	1 : 0.001	24	0.048	1X	5		22.5	0
5	1 : 0.0001	24	0.0048	1X	5		22.5	0

4. Spin down enough G-Actin and Proteins at 100,000g in TLA-100 for 15 min at 6°C to run all of the reactions and controls.
5. Mix G-actin to determined concentration with 18 MΩ dH2O and enough 10X Mg Exchange buffer so Mg Exchange buffer is 1X in Final Volume. For example above, need [Actin]=53.3μM in 22.5μL
6. Add protein mix first, actin mix second, and the 5μL 10x F-Buffer last. Mix by pipetting up and down a few times and then quickly draw up into the capillary tube. Seal bottom, label tube, clamp

vertical and let polymerize for specific amount of time. For above example, 30-60 minutes because [actin] is so high, reaction goes to completion quickly.

7. Run experiment at set angle with same types of tubes and balls. Use glycerol and water samples to calculate the ball constant.

UNITS

8. Velocity (v) = cm ball traveled / secs took to travel that length cm/sec
Viscosity (η) = Ball constant / v mPas*sec
Ball constant for glycerol = $v * 1408\text{mPas*sec}$ mPas*cm
Ball constant for water = $v * 1\text{mPas*sec}$ mPas*cm

9. Record length & time. Calculate velocity and viscosity. Plot on a log x axis to determine which range to narrow the testing to. Repeat experiments with different concentrations as needed.

FIXING *DICTY* CELLS W/ PARAFORMALDEHYDE

PREP

1. Thaw 15mL tube of 16% paraformaldehyde.
2. Sterilize cover slips in a beaker of ethanol. Set up and label as many 6 well plates as need.
3. Carefully remove a cover slip with tweezers. Blot a corner on a Kimwipe and put cover slip quickly through a flame to burn off rest of ethanol. Once flame is out, put cover slip into a well. Repeat for as many samples as need.
4. Resuspend cells and add 1mL cells to each well. Add 2mL fresh media to wells to dilute. Let cells sit at room temperature for a few hours to adhere onto coverslip.
5. Make Fix Mix (~2mL for each sample) Make Immunofluorescence 1X PBT

4% paraformaldehyde	1X PBS
0.1% Triton X-100	0.05% Triton X-100
150mM NaCl	0.5% BSA
6. For 100mL 1X Immunofluorescence PBT
10mL 1X PBS
500 μ L 10% Triton X-100
0.5g BSA

FIXING

7. When cells are ready, take off media carefully and add 2mL Fix Mix very gentle to side of well so that the cells are not blasted off of the cover slip.
8. Leave Fix Mix on for 5 min.
9. After 5 min., take off Fix Mix and immediately add 2mL 1X PBS to each well. Take off and add 2mL fresh 1X PBT again. Repeat this wash 4-5 times for each well.
10. Mount cover slips on slides
Add 10 μ L 20mM Tris + 90% glycerol to the slide where placing the cover slip.
Use tweezers to grab the cover slip, blot corner on a Kimwipe and put on slip,
Make sure that cell side is down.

ACETONE FIX

Add 2mL 100% chilled acetone to each well after removing the media. Put wells on ice for 3 min. Remove acetone and start washes.

FREEZING *DICTYOSTELIUM* CELLS

*Need: A bucket of ice and 2mL Freeze Mix per confluent plate.
Each plate of cells makes 4 aliquots to freeze.*

1. Mix amount of Freeze Mix needed. mL needed = (Z plates x 3) Invert a few times. Reaction is exothermic, will feel a little warm. Immediately put on ice and chill at least 15 minutes.
2. Label as many screw top 1.5mL tubes as needed. Put clear tape on top of label to keep it from fading. Label top of tube with cap label.
3. Pour media off of plates.
4. Take 2mL freeze mix and resuspend cells quickly.
5. Add 500mL aliquots to each tube and immediately put on ice.
6. Store cells in -80°C freezer.
7. Fill out Storage Log for new tubes.

Freeze Mix

10% DMSO in Bis-Tris HL-5 pH 6.7

To Thaw

1. Remove tube from -80°C freezer.
2. Warm in hand as you walk to work area.
3. Wash exterior of tube with ethanol and wipe with kimwipe.
4. Open tube and pipet contents into a 100mm plate containing 10mL of media.
5. If not completely thawed, rinse tube with media from plate and transfer contents.
6. Change media after approx. 15 minutes.
7. Take 1 mL off and add to new plate with 9mL media to dilute DMSO 10-fold.